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Interleukin-12-Stimulated Natural Killer Cells Can Activate Human Macrophages To Inhibit Growth of *Mycobacterium avium*

LUIZ E. BERMUDEZ,* MARTIN WU, AND LOWELL S. YOUNG

Kuzell Institute for Arthritis and Infectious Diseases, California Pacific Medical Center Research Institute, San Francisco, California 94115

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Interleukin-12 (IL-12) is a critical cytokine that affects many of the biological functions of NK cells and T cells. We have previously shown that both human and murine NK cells are important in host defense against *Mycobacterium avium* complex and act by secreting cytokines that induce macrophages to inhibit the growth of intracellular *M. avium*. To define the role of IL-12 in *M. avium* complex infection, we stimulated human NK cells with recombinant human IL-12 at 0.01 to 1 ng/ml for 24 h and used the tissue culture supernatant to treat human monocyte-derived macrophage monolayers infected with *M. avium*. IL-12 had no direct effect on *M. avium*-infected macrophages, but culture supernatant from IL-12-treated NK cells activated macrophages to inhibit the growth of intracellular *M. avium* in a dose-dependent manner. Stimulation of NK cells with IL-12 in combination with tumor necrosis factor alpha (TNF-α) or IL-1 increased the ability of supernatant from NK-cell culture to limit *M. avium* growth within macrophages, compared with that of culture supernatant from IL-12-treated NK cells. Results with supernatant from nonstimulated NK cells were similar to those with supernatant from untreated controls. Treatment of supernatant from IL-12-stimulated NK cells with anti-TNF-α, anti-granulocyte-macrophage colony-stimulating factor, but not anti-gamma interferon antibodies decreased the ability of NK-cell supernatant to induce anti-*M. avium* activity in infected macrophages. Treatment of macrophage monolayers with anti-transforming growth factor β antibody before adding supernatant from IL-12-stimulated NK cells was associated with an increase of anti-*M. avium* activity compared with that of supernatant from IL-12-treated NK cells. These results suggest that IL-12 has a role in host defense against *M. avium* and that the effect of IL-12 is dependent chiefly on TNF-α and granulocyte-macrophage colony-stimulating factor.

Disseminated infection caused by organisms of the *Mycobacterium avium* complex occurs commonly in patients with advanced stages of AIDS (24, 33). *M. avium* is an intracellular pathogen that preferentially infects mononuclear phagocytes. Macrophages seem to be an essential component of the immune response to mycobacteria, but inhibition of growth or killing of virulent strains of mycobacteria requires macrophage activation by cytokines (5, 15, 34).

The mechanisms of host defense against *M. avium* are poorly understood, but there is evidence that $CD4^+$ T cells, $CD8^+$ T cells, and NK cells participate. For instance, a number of laboratories have demonstrated the participation of both T-cell-dependent and -independent host defenses against *M. avium* (8, 9, 14, 27). In addition, the role of NK cells in the early, nonspecific immune response has been suggested in both in vitro and in vivo studies (4, 8, 9, 22, 26).

Interleukin-12 (IL-12), originally known as NK-cell stimulatory factor, is a heterodimeric cytokine regulating many of the biological functions of NK cells and T cells (16, 28, 30). IL-12 is produced mainly by macrophages and B lymphocytes in response to bacterial and parasitic antigens. Furthermore, it can promote the development of Th1 cells in vitro and in vivo (29, 35, 36).

In this report, we describe the effects of IL-12 alone and in combination with IL-1β, tumor necrosis factor alpha (TNF-α), IL-10, and transforming growth factor β (TGF-β) on NK-cell-mediated mycobacteriostatic activity of human macrophages.

* Corresponding author. Mailing address: Kuzell Institute for Arthritis and Infectious Diseases, 2200 Webster St., Rm. 305, San Francisco, CA 94115.

MATERIALS AND METHODS

Mycobacteria. *M. avium* 101 (serovar 1) was isolated from a patient with AIDS and cultured as previously described (5). Mycobacteria were cultured in Middlebrook 7H10 agar (Difco, Detroit, Mich.) for 10 days at 37°C. Transparent colonies were resuspended in Hanks' balanced salt solution (HBSS) and washed twice, and the final suspension was vortex agitated for 2 min. The bacterial suspension was allowed to rest for 5 min, after which the top 2 ml was obtained. Light microscopy observation showed that it was constituted primarily of dispersed bacilli. The suspension was then adjusted to 10^7 bacteria per ml by using a McFarland turbidity standard. A sample from the bacterial suspension was plated onto Middlebrook 7H10 agar for confirmation of the inoculum.

Cytokines. Recombinant human IL-12 was kindly provided by Genetics Institute, Cambridge, Mass. The cytokine was kept at −70°C, and appropriate concentrations were prepared before each experiment. The cytokine specific activity was 10^5 U/mg of protein. Recombinant TNF-α was a gift from Genentech, Inc., South San Francisco, Calif. Recombinant IL-10 was provided by DNAX, Palo Alto, Calif., and had a specific activity of 10^5 U/mg of protein. Recombinant human IL-1β was purchased from Genzyme, Cambridge, Mass., and had a specific activity of 10^6 U/ml of protein. Recombinant TGF-β1 was purchased from R and D Systems, Minneapolis, Minn., and had a specific activity of 5×10^5 U/mg of protein. Recombinant human IL-2 was kindly provided by Chiron, Berkeley, Calif. Endotoxin contamination was ruled out by measurement of the endotoxin concentration by the *Limulus* amebocyte lysate assay (Sigma Chemical Co., St. Louis, Mo.) as well as by using heat-inactivated cytokine preparations. The fetal bovine serum (FBS) used was endotoxin tested and did not stimulate either antimycobacterial activity in *M. avium*-infected macrophages or production of macrophage-stimulating cytokines by NK cells (data not shown).

Human monocyte-derived macrophages. Heparinized human peripheral venous blood was obtained from healthy donors (five donors were used for all the reported experiments), processed by centrifugation with Ficoll-Hypaque (Sigma Chemical Co.), and cultured by previously described techniques (5, 7). The suspension was adjusted to 10^7 mononuclear phagocytes in RPMI 1640 supplemented with 5% heat-inactivated FBS (Sigma) (56°C for 30 min) and then

TABLE 1. IL-12-dependent mycobacteriostatic activity of human macrophages

Treatment (IL-12 concn)	Concn (μg/ml)	No. of viable bacteria at day 4[a]	% Reduction
None		$(9.7 \pm 0.3) \times 10^6$	
IL-12	0.01	$(9.6 \pm 0.4) \times 10^6$	0
	0.1	$(9.3 \pm 0.2) \times 10^6$	4.1
	1	$(8.7 \pm 0.4) \times 10^6$	10.3
Supernatant from NK cells[b]	0.1	$(9.8 \pm 0.3) \times 10^6$	0
	1	$(9.6 \pm 0.4) \times 10^6$	0
Supernatant from IL-12-treated NK cells (0.01 ng/ml)[b]	0.1	$(9.2 \pm 0.2) \times 10^6$	5.1
	1	$(8.0 \pm 0.3) \times 10^6$	17.5
Supernatant from IL-12-treated NK cells (0.1 ng/ml)[b]	0.1	$(4.1 \pm 0.4) \times 10^5$	54.7[c]
	1	$(1.2 \pm 0.3) \times 10^5$	87.6[c]

[a] Number of organisms at time 0 (before treatment), $(7.2 \pm 0.3) \times 10^5$.
[b] Monolayers were treated with supernatants daily for 4 days.
[c] $P < 0.05$ compared with untreated control.

distributed in 1-ml aliquots into 24-well tissue culture plates (Costar, Cambridge, Mass.). The plates were incubated for 2 h at 37°C under 5% CO_2 in moist air to permit adherence of monocytes. Medium and nonadherent cells were then removed by aspiration, and the number of cells in the monolayers, which contained approximately 10^6 monocytes, was determined by the method of Nakagawara and Nathan (32). Monolayers were washed twice at 37°C with HBSS to remove nonadherent cells. The adherent monocytes were maintained in culture for 7 days with 1.5 ml of RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine. These cells developed the morphological criteria for macrophages in approximately 4 days. About 20 to 40% of culture macrophages detached from the plates (experimental and controls) during the first 7 days. Subsequently, 1 to 2% of the cells in both groups (activated and nonactivated) were lost every 2 days. More than 96% of the cells in the monolayers were mononuclear phagocytes, as assessed by their ability to ingest neutral red.

Purification of NK cells. NK cells were purified by discontinuous Percoll gradient centrifugation as previously reported (8). Briefly, Percoll (Sigma) was made isosmotic by the addition of 10% (vol/vol) 10-fold-concentrated HBSS and added in various proportions to culture medium to produce solutions that ranged from 31 to 54% Percoll. The resulting seven solutions were carefully layered in a 15-ml polystyrene tube (Falcon). Nonadherent mononuclear cells, after 2 h of plastic adherence, were passed twice through a sterile nylon wool filter for 1 h each to remove B lymphocytes, and the suspension was incubated with anti-CD4 and anti-CD8 antibodies (50 μg/ml; Becton-Dickinson, Mountain View, Calif.) in the presence of serum. The final suspension was loaded onto the top layer of the Percoll and subsequently centrifuged at $550 \times g$ for 30 min at room temperature. Cells from the second and third interfaces were carefully removed with a Pasteur pipette and washed three times with HBSS. After depletion of high-affinity peripheral rosette-forming erythrocytes, the lymphocyte preparation was adjusted to 10^6 lymphocytes per ml and examined for purity. The preparations were composed of approximately 90 to 95% large granular lymphocytes as assessed by Giemsa stain and by indirect immunofluorescence with an anti-human Leu-11a monoclonal antibody (Becton-Dickinson). The presence of contaminant T cells was also examined by indirect immunofluorescence with anti-human CD4 and CD8 monoclonal antibodies (Becton-Dickinson). Less than 1% contaminant T cells were observed in the preparations.

Macrophage infection. Monocyte-derived macrophages (approximately 10^6 cells per ml), cultured in vitro for 7 days, were incubated with a suspension of *M. avium* (10^7 bacteria per ml) in RPMI 1640 supplemented with 5% heat-inactivated FBS and 2 mM L-glutamine. After 4 h, the extracellular bacilli were removed by extensive washing with HBSS. The number of viable intracellular bacilli was then determined after lysis of the macrophage monolayers. The lysate was cultured onto Middlebrook 7H10 agar plates as described below to establish the number of phagocytosed bacteria per monolayer (baseline inoculum). The percentage of infected macrophages in the monolayers was 76% ± 6% at the beginning of the experiment and 81% ± 4% by the end of the experiment.

NK cell supernatant. Activated and nonactivated NK cells were cultured in RPMI 1640 plus 5% FBS. After 24 h, the supernatant was removed and centrifuged at $5,000 \times g$ for 15 min. The recovered supernatant was filtered through a 0.2-μm-pore-size Millipore filter and stored at −70°C until the moment of the experiment. The amount of protein in the supernatant was measured by a protein assay (Bio-Rad Laboratories, Hercules, Calif.).

Neutralizing antibodies. Mouse anti-human TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and gamma interferon (IFN-γ) antibodies were obtained from Genentech; Immunex Corp., Seattle, Wash.; and Genzyme, respectively. All antibodies were used at a concentration able to neutralize 10^4 U of recombinant cytokine. Irrelevant antibody (mouse anti-*Pseudomonas aeruginosa* immunoglobulin G and PCB5) was used as negative control. The ability of the above antibodies to neutralize TNF-α, GM-CSF, or IFN-γ was established in preliminary experiments with increasing concentrations of the antibodies (data not shown).

Quantitation of intracellular bacteria. Inhibition of growth or killing of intracellular bacteria was concluded to have occurred when the number of bacteria in stimulated monolayers was smaller than in control monolayers at the same time point after infection, considering only monolayers with a similar number of bacteria at time zero after infection. Macrophage monolayers were infected with *M. avium* and cultured at 37°C under 5% CO_2 in moist air for several days. To lyse macrophages, 0.5 ml of iced sterile water was added to each well and tissue culture plates were maintained for 10 min at room temperature. Then, 0.5 ml of another lysing solution containing 1.1 ml of 7H9 medium and 0.4 ml of 0.25% sodium dodecyl sulfate (SDS) in phosphate buffer was added to each well for another 10 min. The wells were vigorously scraped with a rubber policeman, and the macrophage lysates were resuspended in 0.5 ml of 20% bovine serum albumin in sterile water to neutralize the effect of SDS. The suspension was then vortex agitated for 2 min to effect complete lysis of macrophages. The macrophage lysate was briefly sonicated for 5 s (power output, 2.5 W/s) to decrease bacterial clumping and permit reproducible pour plate quantitation. As a control for osmotic stability, mycobacteria without macrophages were subjected to the same procedure and quantitative colony counts were determined by pour plate methods. The bacteria were 100% viable. To ensure that macrophages were totally disrupted, samples were examined by Giemsa staining.

The macrophage lysate suspension was serially diluted, and 0.1 ml of the final suspension was plated onto 7H10 agar. The plates were allowed to dry at room temperature for 15 min and incubated at 37°C for 2 weeks. The results are reported as mean CFU per milliliter of macrophage lysate. Duplicate plates were prepared for each well.

Statistical analysis. Each experiment was repeated at least three times. All data in each assay were obtained in a duplicate well, and the means and standard deviations were calculated. The significance of the differences in results between control and experimental groups was determined by Student's *t* test.

RESULTS

Effect of recombinant IL-12 and NK-cell supernatant. To evaluate whether IL-12 had any direct stimulatory effect on human macrophages, macrophage monolayers infected with *M. avium* 101 were treated for 4 days with IL-12 at 0.01, 0.1, 1, 10, and 100 ng/ml and 1 μg/ml. As shown in Table 1, IL-12 had no direct stimulatory effect on infected macrophages. Then we sought to detect any effect of culture supernatant obtained from NK cells treated with 0.01 or 1 ng of IL-12 per ml in the *M. avium*-infected macrophages. NK cells were treated with IL-12 (0.01 and 0.1 ng/ml), and after 24 h the NK cells were pelleted and supernatant was obtained. The concentration of protein in the culture supernatant was determined, and concentrations of 0.1 and 1 μg/ml were used to stimulate *M. avium*-infected macrophages.

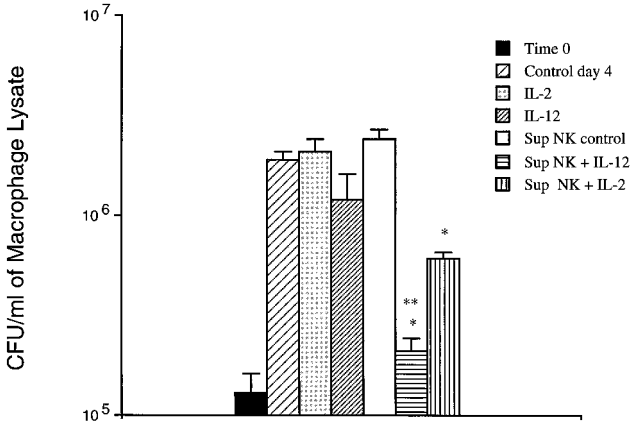

FIG. 1. Effect of NK cell supernatant on the ability of macrophages to inhibit the intracellular growth of *M. avium*. NK cells were incubated for 24 h with 0.1 ng of recombinant IL-12 or IL-2 per ml. Supernatant (Sup) was then obtained, and 1 μg/ml was used to treat *M. avium*-infected macrophage monolayers for 4 days. Control macrophage monolayers treated with recombinant IL-2 (0.5 ng/ml), IL-12 (0.5 ng/ml), and supernatant of unstimulated NK cells were run in parallel. *, $P < 0.05$ compared with untreated control. **, $P < 0.05$ compared with Sup NK + IL-2.

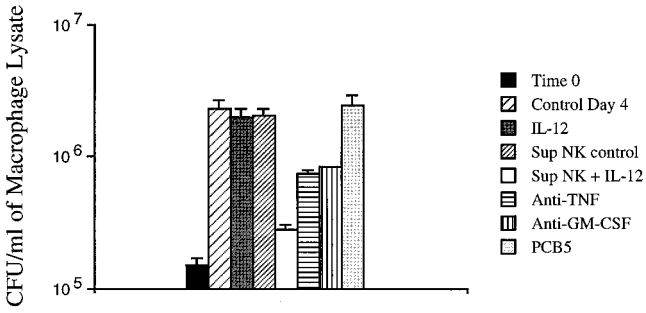

FIG. 2. Inhibition of the mycobacteriostatic effect of supernatant from IL-12-treated NK cells by the addition of anti-TNF-α and anti-GM-CSF. NK cells were treated with IL-12 (0.1 ng/ml) for 24 h, and the supernatant (Sup) was obtained. Macrophage monolayers were incubated with anti-TNF-α monoclonal antibody (sufficient to inhibit 10^4 U of TNF-α per ml) or anti-GM-CSF monoclonal antibody (sufficient to inhibit 10^4 U of TNF-α per ml) prior to the treatment with supernatant from NK cells (1 μg/ml). Recombinant IL-12 and supernatant of unstimulated NK cells were used as controls. Anti-TNF-α and anti-GM-CSF antibodies had no effect on the number of *M. avium* organisms within macrophages (data not shown). An anti-*Pseudomonas aeruginosa* antibody (PCB5) was used as an irrelevant control.

As shown in Table 1, culture supernatant obtained from IL-12-treated NK cells activated macrophages to inhibit the intracellular growth of *M. avium* in a dose-dependent fashion.

Comparison of the effects of IL-12 and IL-2. Previous studies have demonstrated that culture supernatant from IL-2-treated NK cells was also able to stimulate human macrophages to inhibit the intracellular growth of *M. avium* (8). Therefore, we compared the effects of culture supernatants from NK cells treated with three different concentrations of IL-2 and IL-12 for their ability to stimulate mycobacteriostatic activity in human macrophages. As shown in Fig. 1, culture supernatants from NK cells stimulated with either IL-12 or IL-2 induced significant inhibition of growth of intracellular *M. avium*. Treatment of monolayers with culture supernatant from IL-12-stimulated NK cells resulted in significantly greater mycobacteriostatic activity than did IL-2-dependent NK-cell activity. For these experiments, the graph shows only the most efficacious concentrations.

Effect of anti-TNF-α, anti-GM-CSF, and anti-IFN-γ antibodies. Because TNF-α, IFN-γ, and GM-CSF, three cytokines produced by NK cells, were shown in previous studies to stimulate mycobacteriostatic activity in macrophages (5, 9, 37), we sought to determine whether incubation with neutralizing antibodies to TNF-α, IFN-γ, and GM-CSF was associated with inhibition of NK-cell-dependent mycobacteriostatic activity in macrophages. As shown in Fig. 2, anti-TNF-α and anti-GM-CSF antibodies significantly inhibited the mycobacteriostatic activity supernatant from NK-cell culture, although complete suppression was not observed. Table 2 shows that the presence of anti-IFN-γ antibody did not have any effect on NK-cell supernatant-dependent anti-*M. avium* activity. Treatment with antibodies by themselves had no effect on *M. avium* growth either extracellularly or intracellularly.

Combination of IL-12 with TNF-α. TNF-α stimulates NK cells to produce IFN-γ (12). To determine whether NK cells treated with TNF-α alone or in combination with IL-12 could induce greater mycobacteriostatic activity in macrophages than could supernatant from IL-12-treated NK cells, purified NK cells were incubated with recombinant TNF-α (10^2 U/ml) for 8 h, washed, and subsequently incubated with IL-12 for 24 h. Culture supernatant was then obtained and used to stimulate *M. avium*-infected macrophages. As shown in Fig. 3, pretreatment of NK cells with TNF-α significantly increased the IL-12-mediated activation of NK cells.

Because NK cells stimulated with IL-12 and TNF-α have been shown to produce and secrete increased amounts of IFN-γ when compared with NK cells treated with IL-12 alone, the above-described experiment was repeated with anti-IFN-γ monoclonal antibody (Genzyme) to determine how much of the effect of IL-12 and TNF-α was due to the production of IFN-γ. As shown in Table 2, incubation of infected macrophages with supernatant from NK-cell culture in the presence of anti-IFN-γ antibody did not have a significant blocking effect on the mycobacteriostatic activity (approximately 14%).

Effect of treatment with IL-1β. We had recently demonstrated that the combination of IL-1β and IL-12 is capable of inducing NK cells to produce a significantly greater concentration of IFN-γ than is IL-12 alone (25). On the basis of this observation, we treated NK cells with IL-1β (10^2 U/ml) for 8 h and then, following the removal of supernatant, exposed NK cells to IL-12 (0.1 ng/ml) for 24 h. The culture supernatant was then used to stimulate *M. avium*-infected macrophage mono-

TABLE 2. Effect of anti-IFN-γ antibody on the mycobacteriostatic activity of macrophages stimulated with supernatant from TNF-α- and IL-12-treated NK cells[a]

Treatment	No. of viable bacteria at day 4[b]	% Reduction
None	$(4.0 \pm 0.4) \times 10^6$	
Supernatant from NK cells[c]	$(2.9 \pm 0.3) \times 10^6$	27
Supernatant from NK cells + IL-12 (0.1 ng/ml)[c]	$(3.6 \pm 0.4) \times 10^5$	91[d]
Supernatant from NK cells + TNF-α (10^2 U/ml) + IL-12 (0.1 ng/ml)[c]	$(8.5 \pm 0.4) \times 10^5$	97.8[d]
Supernatant from NK cells + TNF-α (10^2 U/ml) + IL-12 (0.1 ng/ml) + anti-IFN-γ[c]	$(6.1 \pm 0.3) \times 10^5$	84.7[d]

[a] NK cells were treated with TNF-α (10^2 U/ml) for 8 h and then with IL-12 (0.1 ng/ml) for 24 h.
[b] Number of intracellular *M. avium* at time zero, $(3.4 \pm 0.3) \times 10^5$.
[c] Monolayers were treated with supernatants for 4 days.
[d] $P < 0.05$ compared with untreated control.

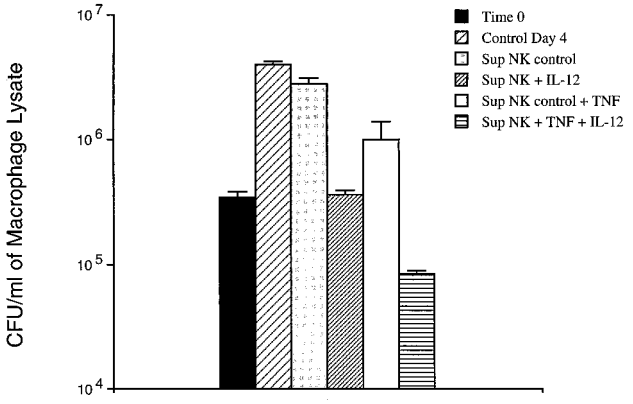

FIG. 3. Ability of TNF-α in combination with IL-12 to induce NK-cell supernatant (Sup) to stimulate mycobacteriostatic activity in macrophages. Human NK cells were incubated with TNF-α for 8 h, washed, and subsequently incubated with IL-12 for 24 h. Appropriate controls were run in parallel. Treatment of NK cells with TNF-α and IL-12 was associated with a significant increase in mycobacteriostatic activity of macrophages treated with NK supernatants when compared with the use of IL-12 or TNF-α alone.

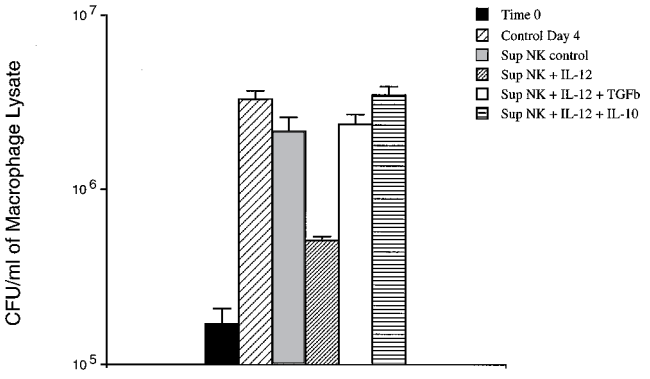

FIG. 4. Effect of pretreatment with TGF-β or IL-10 on the ability of IL-12 to stimulate NK cells. NK cells were treated with TGF-β (10^2 U/ml) or IL-10 (10^2 U/ml) for 8 h, prior to treatment with IL-12 (0.1 ng/ml). Sup, supernatant.

layers. As shown in Table 3, culture supernatants derived from NK cells treated with the combination of IL-1β and IL-12 resulted in consistent but not significant augmentation of mycobacteriostatic activity compared with culture supernatants from NK cells treated with either IL-12 or IL-1 alone.

Effect of TGF-β and IL-10. Because both TGF-β and IL-10 can suppress NK-cell activity, we examined the effect of both recombinant TGF-β and IL-10 on the ability of IL-12 to stimulate NK cells. NK cells were treated with TGF-β (10^2 U/ml) or IL-10 (10^2 U/ml) for 8 h, and then the supernatant was removed and IL-12 (0.1 ng/ml) was added for 24 h. Culture supernatant obtained from NK-cell cultures and 1 μg of protein per ml was used to treat *M. avium*-infected macrophages. As shown in Fig. 4, both TGF-β and IL-10 significantly inhibited the ability of IL-12 to stimulate NK cells.

DISCUSSION

This report provides evidence that recombinant IL-12 stimulates NK-cell-dependent induction of antimycobacterial activity in macrophages. We showed that treatment of human NK cells with recombinant IL-12 resulted in the production in the culture supernatant of soluble factors that triggered human macrophages to inhibit intracellular growth of *M. avium* in a dose-related manner.

M. avium is an intracellular pathogen that survives within macrophages (15). The overwhelming majority of individuals who encounter *M. avium* (whose sources are widespread in the environment) never develop disseminated disease, suggesting that effective mechanisms of defense exist. In contrast, immunocompromised patients, in particular those with AIDS, are more susceptible to infection and eventually to development of disease (24, 33). Activation of macrophages by cytokines has been shown in vitro and in vivo to be associated with inhibition of growth and killing of intracellular *M. avium* (5, 7). In addition, $CD4^+$ T cells and NK cells were shown in a number of in vitro and in vivo models to play a beneficial role in the host immune response against *M. avium* (1, 4, 5, 8, 10, 14, 22, 26, 27, 34).

TABLE 3. Effect of IL-1β on the IL-12-dependent mycobacteriostatic activity

Treatment	No. of viable bacteria at day 4[a]	% Reduction
None	$(5.3 \pm 0.3) \times 10^6$	
NK control[b]	$(3.8 \pm 0.5) \times 10^6$	28
NK + IL-12 (0.1 ng/ml)[b]	$(9.0 \pm 0.4) \times 10^5$	83[c]
NK + IL-1 (10^2 U/ml)[b]	$(4.6 \pm 0.3) \times 10^6$	13
NK + IL-1 (10^2 U/ml) + IL-12 (0.1 ng/ml)[b]	$(6.8 \pm 0.3) \times 10^5$	87[c]

[a] Number of bacteria at time zero, $(4 \pm 0.4) \times 10^5$.
[b] Supernatants were added for 4 days.
[c] $P < 0.05$ compared with untreated control.

IL-12 is a recently described cytokine that appears to play a central role in the immune response against infectious organisms such as *Leishmania* species and *Toxoplasma gondii* (1, 23, 29, 35). IL-12 has been recently shown to be produced by mononuclear cells obtained from the pleural fluid of patients with tuberculosis, suggesting that IL-12 participates in the antimycobacterial immune response (40). Our study, however, showed that IL-12 does not act directly on *M. avium*-infected macrophages. This finding is in agreement with a previous observation that macrophages and monocytes do not express specific receptors for IL-12 (38). In contrast, when NK cells were exposed to recombinant IL-12 and culture supernatant was used to stimulate *M. avium*-infected macrophages, antimycobacterial activity was observed. In previous studies, we determined that recombinant IL-2 could also induce NK-cell-mediated mycobacteriostatic activity in macrophages (8). Comparison of the ability of IL-2 and IL-12 to induce NK-cell-mediated activation of macrophages showed that IL-12 was significantly more active than IL-2. IL-2 is a potent stimulator of the proliferation of NK cells and may act together with IL-12 to orchestrate the immune response. In mice with severe combined immunodeficiency, the IL-2 signal is never present, because of the T-cell deficiency, and the animals are unable to clear *Listeria monocytogenes* (39) or restrict *T. gondii* replication (21, 25). However, the ability of large doses of IL-12 to slow *T. gondii* replication demonstrates that IL-12 has some ability to overcome the loss of IL-2 in immunodeficient hosts (39).

IL-12 stimulated NK cells to secrete TNF-α, GM-CSF, and IFN-γ. Use of monoclonal antibodies in combination with NK-cell-derived culture supernatant demonstrated that NK-cell-mediated antimycobacterial activity of macrophages was due in part to TNF-α and GM-CSF but not IFN-γ. However, antibodies against TNF-α and GM-CSF could not completely block the effect of NK-cell culture supernatant. Assays with

anti-TNF-α and anti-GM-CSF in combination did not give reproducible results, probably because of interaction of the antibodies. TNF-α, GM-CSF, and IFN-γ have been associated with murine and human immune responses to *M. avium* infection in vitro and in vivo (5, 7, 17, 37). The effect of TNF-α and GM-CSF on *M. avium*-infected macrophages appears to be dependent on the load of bacteria and on the period of infection (6). Recently infected macrophages are fully responsive to stimulation with TNF-α and GM-CSF, whereas macrophages with large numbers of intracellular *M. avium* organisms seem to be refractory to these cytokines. It has been proposed that NK cells can respond to mycobacteria early in the course of infection, perhaps as a nonspecific mechanism of defense (9, 22). According to this hypothesis, one would assume that once infected with *M. avium*, macrophages would secrete IL-12, triggering cytokine production by NK cells. Macrophages exposed to endotoxin transcribe TNF-α and IL-12 synthesis relatively rapidly, with a peak around 4 to 8 h (35), and costimulation of NK cells with recombinant IL-12 and TNF-α resulted in increased antimycobacterial activity of macrophages.

Recently, Tripp et al. (39), examining the immune response against *L. monocytogenes* in mice with severe combined immunodeficiency, also observed that TNF-α acted synergistically with IL-12 to augment the production of IFN-γ by NK cells. Although treatment of NK cells with TNF-α in combination with IL-12 resulted in augmented NK-cell-mediated anti-*M. avium* activity in macrophages, our result of assays with monoclonal antibodies to neutralize the effect of cytokines showed that IFN-γ had no significant participation in NK cell-mediated anti-*M. avium* activity. The ability of IFN-γ to induce anti-*M. avium* activity in human macrophages appears to be strain dependent and is inversely related to the ability of the *M. avium* strain to induce the production of TGF-β by macrophages (2).

Our results showing that NK cells treated with IL-1β and IL-12 can induce an augmented antimycobacterial activity by NK cells treated with IL-12 are intriguing. IL-1β is a proinflammatory cytokine that is produced mostly by mononuclear cells (20). Although IL-1β has not been shown to be a potent stimulator of NK-cell function, recent work by Hunter et al. found that IL-1β potentiates the effect of IL-12 on NK cells to enhance the production of IFN-γ (25). Furthermore, a recent study by Denis and Ghadirian (19) demonstrated that although IL-1β had no direct antimycobacterial effect on *M. avium*-infected macrophages, mice treated with recombinant IL-1β were able to clear *M. avium* infection more efficiently than were controls. It is not known if IL-1β had any stimulatory effect on NK cells of *M. avium*-infected mice.

Studies from our and other laboratories have shown that human macrophages infected with virulent mouse strains of *M. avium* secrete large amounts of active TGF-β (2). In addition, murine infection with *M. avium* is associated with increased levels of IL-10 and TGF-β both in serum and in splenic cell culture supernatant (3, 18). Both IL-10 and TGF-β are known to suppress a number of macrophage functions, including mycobacteriostatic activity (11, 13). Recent studies have demonstrated that IL-10 is a physiologic antagonist of IL-12 (39) and can suppress most of the effects of IL-12 in T cells and NK cells (31). Anti-IL-10 antibodies have been shown to increase the ability of IL-12 to induce a Th1-type response in mononuclear lymphocytes from AIDS patients with tuberculosis (41). Furthermore, TGF-β is a potent inhibitor of IL-12-mediated stimulation of NK cells (25). Our results showing that NK cells exposed to TGF-β prior to stimulation with IL-12 have impaired ability to induce mycobacteriostatic activity in macrophages support these observations.

Recently, Zhang et al. reported on the presence of IL-12 at the site of tuberculosis pleuritis (41). IL-12 was produced by pleural fluid cells in response to *M. tuberculosis*, and anti-IL-12 antibodies inhibited *M. tuberculosis*-induced proliferation of pleural lymphocytes, suggesting that IL-12 contributes an antigen recognition by T cells. IL-12 may also enhance antimycobacterial immune defenses by stimulating NK cells. NK cells have been shown to enhance antimycobactericidal activity of macrophages in a number of systems (4, 8, 9, 14, 22, 26, 27) and to increase in number during mycobacterial infections (4, 8, 9, 14, 22, 26, 27).

In summary, we have shown that IL-12 can induce NK-cell-mediated mycobacteriostatic activity in human macrophages and that IL-12 has the potential to contribute to immune defenses against mycobacterial infections. Further investigations should help in clarifying the role of IL-12 in vivo and its potential as an immunoregulatory agent.

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